Research into the protective effect of syringic acid in rats with induced experimental pancreatitis

Protective effect of syringic acid on pancreatitis

Mehmet Sertkaya[1], Fatih Mehmet Yazar[2], Ömer Faruk Boran[3], Aykut Urfalıoğlu[3], Abdulkadir Yasir Bahar[4], Hasan Dağlı[5], Seda İkikardeş[6], Metin Kılınç[7]
[1]Department of General Surgery, Adıyaman University Research and Training Hospital, Adıyaman,
[2]Department of General Surgery, Faculty of Medicine, Sutcu İmam University, Kahramanmaras,
[3]Department of Anesthesiology and Reanimation, Faculty of Medicine, Sutcu İmam University, Kahramanmaras,
[4]Department of Pathology, Faculty of Medicine, Sutcu İmam University, Kahramanmaras,
[5]Department of Medical Biochemistry, Faculty of Medicine, Sutcu İmam University, Kahramanmaras,
[6]Department of Pathology, Faculty of Medicine, Sutcu İmam University, Kahramanmaras,
7Department of Medical Biochemistry, Faculty of Medicine, Sutcu İmam University, Kahramanmaras, Turkey

Abstract

Aim: The aim of this study was to evaluate the protective effects of dietary syringic acid (SA) on the development and severity of cerulein-induced acute pancreatitis (AP) in rats in terms of biochemical and histopathologic changes. Material and Method: 24 rats were divided into 3 equal groups. Three groups were compared in terms of the levels of oxidative stress, antioxidant markers, pancreatic enzymes, and histopathological changes. This study was approved by the ethics committee of our university and the project was supported by the scientific research unit (project number 2016 / 3-43 D). Results: In the pancreatitis group, the amylase and lipase values were significantly higher and oxidative stress markers were significantly elevated; the Glutathione peroxidase (GSH-Px) level was found to be significantly decreased in the pancreatitis group compared to the control and treatment groups. Apoptosis and cellular damage level results were significantly higher in the pancreatitis group, while cellular damage in the treatment group was significantly lower (P < 0.05) than in the pancreatitis group. Discussion: Although a syringic acid diet does not totally prevent AP, it has been shown to decrease the severity of AP on both enzymatic and histopathological levels.

Keywords

Acute pancreatitis, Syringic Acid, Antioxidant treatment, Phenolic compounds, Cerulein

Introduction

Syringic acid (SA) is a phenolic compound that acts pharmacologically as an antioxidant to remove free radicals in the body [1]. SA is a natural phytochemical derived from Isatis indigotica and Radix isatidis and endows multipharmacologic properties, such as strong antioxidant, antiproliferative, antiendotoxic, antimicrobial, anti-inflammatory, and anticancer effects [2]. Acute pancreatitis (AP) is known to be a consequence of progressive tissue damage at the cellular level in pancreatic tissue due to cytokines and reactive oxygen species (ROS) secreted by inflammatory cells [3,4]. In this study, we aimed to determine whether the dietary intake of SA would prevent AP or mitigate its severity, as shown previously in an experimental model of AP [2]. Our first aim in this study was to investigate the effects of syringic acid on the prevention and the alleviation of acute pancreatitis. Our other aims were to demonstrate the severity of acute pancreatitis induced by cerulein and to investigate by which mechanisms syringic acid prevents and heals it.

Material and Method

Study design and Working Groups

The animal study was conducted at the Experimental Animal Laboratory, Kahramanmaras Sutcu Imam University, after receiving ethics approval from the local Animal Research Ethics Committee. The experimental protocols were carried out according to the ethical principles of the "Institute of Laboratory Animal Resources, Guide for the Care and Use of Laboratory Animals, 8th edition, 2011, The National Academies Press, Washington D.C.". In addition, this project was supported by Kahramanmaras Sutcu Imam University Scientific Research Projects Unit. In the experiment, 24 adult male Wistar Albino rats weighing 300 ± 50 g were used. All rats were housed under standard laboratory conditions, with 22 ± 2° C room temperature, 60 ± 5% moisture, periodic 12-hour light-dark cycles under white fluorescent light and were fed with standard pellet feed (Bil-Yem Lt., Ankara, Turkey). All rats were acclimatized to laboratory conditions for 1 week before starting the experiment. Throughout the experiment, rats were given ad libitum access to food and drinking water. Three groups were formed, each containing 8 rats. During the 1 week acclimatization period, rats in the treatment group were given 50 mg/kg/day SA diluted with 1 mL distilled water via orogastric tube, in addition to standard feed.

Biochemical agents used in the experiment

Syringic acid (SA) 5 g was supplied from Sigma-Aldrich Chemical Co. (St Louis, MO, USA).

Cerulein (Sigma-Aldrich, St. Louis, MO, USA)

Experimental Design

Rats were randomly divided into 3 groups (n = 8). Group 1: Control group (received only intraperitoneal saline).

Group 2: Pancreatitis Group (received 4 × 75 µg/kg cerulein (Sigma-Aldrich, St. Louis, MO, USA) to induce acute pancreatitis; treatment was denied.

Group 3: Treatment Group (received 4 × 75 µg/kg cerulein (Sigma-Aldrich, St. Louis, MO, USA) to induce acute pancreatitis, after being fed for 7 days with 50 mg/kg SA diluted in 1 mL

distilled water via orogastric tube in addition to standard feed. On the 7th day of the study, rats in Groups 2 and 3 received 4 × 75 µg/kg cerulein (Sigma-Aldrich, St. Louis, MO, USA) to induce pancreatitis, while rats in Group 1 received intraperitoneal normal saline of the same volume (0.5cc). (Groups 2 and 3 received cerulein.) 24 hours after the procedure, intracardiac blood samples of about 2.5 cc were obtained and rats were anesthetized with 50 mg/kg ip Ketamine hydrochloride (Ketalar vial, Eczacibasi, İstanbul, Turkey). After shaving, the abdominal skin was disinfected with povidone iodine and a midline incision was made. All pancreatic tissue including the duodenal loop was removed for histopathological examination and tissue antioxidant enzyme activity measurements. At the end of the procedure, the rats were sacrificed by decapitation.

Blood samples were centrifuged at 3000 rpm for 20 min in order to study amylase and lipase levels. Samples were stored at − 80 ° c until analysis.

Preparation of tissue homogenates

In order to perform malondialdehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and catalase (CAT) activity measurement, pancreas tissue samples were homogenized in ice-cold 1.15% KCI solution and centrifuged at 14.000 rpm.

Biochemical Analysis

Determination of MDA, SOD, GSH-Px, CAT levels

Tissue MDA levels were measured by the method of Ohkawa et al. The pink adduct formed by heating MDA and thiobarbituric acid was assayed spectrophotometrically and absorbance was measured at 532 nm [5]. Tissue SOD activity was determined by the degree of inhibition of the reaction by SOD, during the conversion of superoxide radical to hydrogen peroxide. In the reaction, the first 30 seconds of absorbance at 505 nm, at 37°C against air, in cuvettes of 1 cm light path, was measured and SOD activity was calculated. The intensity of the color is inversely proportional to the SOD activity. The method of Sun et al. was used [6]. CAT catalyzes the breakdown of H_2O_2. The breakdown rate of H_2O_2 by CAT was measured spectrophotometrically using the light absorbance rate of H_2O_2 at 230 nm [7]. The Beutler [8] method was used to measure GSH-Px activity. GPx catalyzes the oxidation of reduced glutathione (GSH) to the oxidized glutathione (GSSG) using H_2O_2. Later, GSSG is reduced to GSH by NADPH and glutathione reductase. GPx activity is determined by spectrophotometric measurement of the decrease in absorbance at 340 nm during the conversion of NADPH to NADP.

Detection of Amylase and Lipase levels

Amylase and lipase enzyme levels in serum samples were studied in the biochemistry laboratory in an autoanalyzer (Siemens Advia 1800 Chemistry system, Germany) and the results were quantified according to the calibration curve.

Histopathological Parameters

Specimens were evaluated by an expert pathologist (A.Y.B) who was blinded to the groups according to general H&E diagnostic criteria for apoptosis and necrosis as described by Elmore at

al. [9]. Apoptosis was defined as the morphological features of cell death clearly fit with apoptotic diagnostic criteria. Necrosis was described as the morphological features of cell death clearly fit with necrotic diagnostic criteria. Pancreatic tissue samples were fixed with 10% formaldehyde, then embedded into paraffin blocks and 5 μm sections were prepared using a Leica RM2145 microtome. Tissue sections were stained with hematoxylin-eosin (H&E) and examined under Nikon DS-Fi2 microscope at 400x magnification. Small pyknotic nucleated apoptotic cells with large eosinophilic cytoplasm were counted under 400x magnification, with a conversion factor of 1 mm^2 equaling 4 fields.

Caspase 3 Immunohistochemical Staining

Apoptosis in pancreatic cells and protein expression involved in the regulation of apoptosis were evaluated. Sections prepared by formaldehyde fixation and paraffin blocking were stained immunohistochemically (VentanaBenchMark XT IHC / ISH) with Caspase 3 antibody (BosterBio, 1: 100 dilution). Cells with strong cytoplasmic and nuclear staining indicating high Caspase 3 expression were counted on each slide, at 400 × magnification, in 4 separate zones (approximately 1 mm^2 area).

Statistical Analysis

Statistical analysis was performed using the SPSS 22 software (SPSS® version 22.0; SPSS, Chicago, IL, USA). Numerical variables were presented as Mean ± Standard Deviation (Mean±SD). The groups were compared using ANOVA test. Post-hoc analysis was made with Tukey's test and p <0.05 was considered as significant.

Results

No rats died in any of the groups during the study period.

Biochemical Analysis Results
MDA Values

Compared with the pancreatitis group, the MDA values in Group 1 were significantly lower (P = 0.047). MDA levels in the treatment group were lower than in the pancreatitis group but there was no statistically significant difference (p> 0.05) (Table 1).

CAT Values

Compared with the pancreatitis group, the CAT values in Group 1 and 3 were high, but the difference was not statistically significant (p> 0.05) (Table 1).

SOD Values

Similar to the CAT values, the SOD values in Group 1 and 3 were higher compared with the pancreatitis group, but the difference was not statistically significant (p> 0.05) (Table 1).

GSH-Px values

Compared with the pancreatitis group, GSH-Px values in Group 1 were found to be significantly higher (p= 0.039). Although GSH-Px values in the treatment group were higher than in the pancreatitis group, the difference was not statistically significant (p> 0.05) (Table 1).

Table 1. Comparison between oxidant, antioxidant, and biochemical parameters of groups

	Group 1 (n:8)	Group 2 (n:8)	Group 3 (n:8)	P
GPX(U/mg prt)	0.010±0.003[a]	0.0058±0.0006	0.0082±0.003	0 039
SOD(U/mg prt)	0.0089±0.005	0.0047±0.0007	0, 0061±0.004	0.201
CAT(U/mg prt)	0.414±0.13	0.330±0.08	0.351±0.14	0.461
MDA(nmol/ mg prt)	14.86±7.17[a]	51.81±41.04	29.85±10.88	0,047
Amylase	2012.42±464.69[b]	3135.28±520.63	2487.42±499.63	0. 001
Lipase	10.57±0.78[b]	18.42±4.4	15.71±3.98	0. 001

Data are expressed as the mean±SD (Standard Deviation) unless otherwise noted.
ap<0.05: Group 1 versus Group 2
bp<0.01: Group 1 versus Group 2
cp<0.05: Group 1 versus Group 3
dp<0.01: Group 1 versus Group 3
ep<0.05: Group 2 versus Group3
fp<0.01: Group 2 versus Group 3
GPX:Glutathione peroxidase, SOD:Superoxide dismutase, CAT:Catalase, MDA: Malondialdehyde, U:Unit, mg:milligram, prt:protein, nmol:nanomol

Amylase Values

In the pancreatitis group, amylase levels were found to be statistically significantly higher when compared to the control group (p = 0.001) (Table 1). Although the amylase levels in the treatment group were higher than in the control group, the difference was not significant (p> 0.05).

Lipase Values

It was seen that the lipase levels were similar to the amylase levels. In the pancreatitis group, lipase levels were found to be statistically significantly higher when compared to the control group (P = 0.001) (Table 1). Although the lipase values in the treatment group were low compared to the pancreatitis group, the difference between the groups was not significant (p> 0.05).

Histopathological Results

Histopathological evaluation of apoptotic cells in the pancreas tissue was performed using H&E and Caspase 3 immunohistological staining. Both methods showed that apoptotic cell numbers were significantly lower in the treatment group in comparison to the pancreatitis group (p <0.05). When compared with the control group, apoptosis was found to be significantly higher in both the treatment group (p <0.05) and the pancreatitis group (p <0.001) (Figure 1).
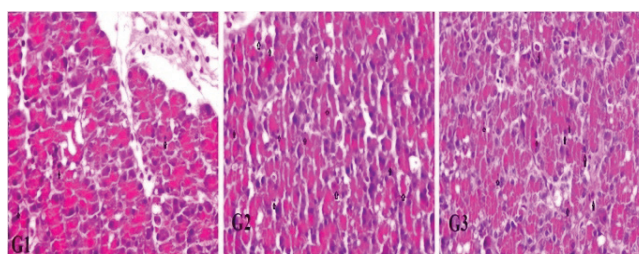

Figure 1. Hematoxylin-eosin (×400) stained sections showing exocrine pancreas tissue sections of Groups 1-2-3. Small dark pyknotic nucleated apoptotic cells with large eosinophilic cytoplasm (arrows) are seen between normal acinar cells.

Table 2. Evaluation of apoptotic cells according to histopathological and immunohistochemical criteria

	Group 1(n:8)	Group 2 (n:8)	Group3 (n:8)	P
H&E stain (original magnification×400)	12.5±1.87[b-c]	36.33±2.16[e]	26.16±1.47	0, 001
Caspase 3 stain (original magnification×400)	14.0±2.36[b-c]	40.16±2.92[e]	28.50±2.73	0.001

Data are expressed as the mean±SD unless otherwise noted.
ap<0.05: Group 1 versus Group 2
bp<0.01: Group 1 versus Group2
cp <0.05: Group 1 versus Group 3
dp<0.01: Group 1 versus Group 3
ep<0.05: Group 2 versus Group3
fp<0.01: Group 2 versus Group 3
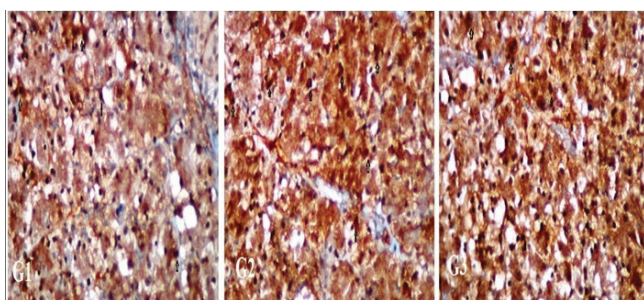H&E: Hemotoxylin and eosin

Figure 2. Sections showing exocrine pancreas tissue. Cells with high Caspase 3 expression show strong positive immunohistochemical staining (arrows) in the cytoplasm and nucleus.

Discussion

The etiology of acute pancreatitis (AP) is still unclear, although recent studies suggest that etiopathogenesis depends on multiple immunological, microbiological, and genetic factors. When the clinical features of the patients are assessed, it is seen that the main cause of pancreatic inflammation is the exaggerated immune response caused by the autolysis of the tissue with pancreatic enzymes, which is triggered by excessive alcohol or fatty food intake [10]. The progressive tissue damage at the cellular level of pancreatic tissue is the result of the cytokines and reactive oxygen species (ROS) secreted by the inflammatory cells [3, 4]. Most studies investigating the early and chronic phases of the disease have emphasized the role of B and T lymphocytes, macrophages, neutrophils, cytokines, inflammation mediators, and reactive oxygen species (ROS) [11-13]. For this reason,   various anti-inflammatory [14,15], antioxidant [4,15,16], and immunomodulatory [17] agents have been used in experimental studies for therapeutic purposes. The main purpose of these previous studies was to increase the activity of antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and catalase (CAT) which prevent inflammation and enable the clearance of ROS [4,16]. The main approach in these studies focused on the treatment of developed disease. However, it is not possible to foresee the degree of inflammation and the outcome once the disease is triggered [3, 4]. This once again underlines the importance of preventive and protective measures.

AP is still a serious disease with no specific treatment and high mortality, despite all improvements in diagnosis and treatment [3, 4]. Our study does not claim that AP treatment is possible with antioxidant agents. On the contrary, the multifactorial na-

ture of the etiology requires a multifactorial treatment. Once the disease is triggered, it is hard to envisage the complications [3, 4]. In addition, we think that the prevention or the mitigation of the severity of the disease is at least as important as treatment.

This study shows that pancreatitis developed both on histopathological and biochemical levels with the administration of intraperitonal cerulein. Though histopathological pancreatitis developed in rats fed with SA prior to the procedure, there was no biochemical difference between the treatment group and the control group. The histopathological difference between the control group and the treatment group proved to be more moderate compared to the difference between the control group and the pancreatitis group.

Cerulein, which is a cholecystokinin analogue, was chosen as the agent to induce pancreatitis because of its ability to mimic edematous pancreatitis biochemically and histopathologically by activating nuclear factor kappaB (NF-κB) which in turn stimulates gastric and pancreatic secretions and causes cytoplasmic vacuolization and edematous pancreatitis [18]. The evaluation of AP development in our study was made objectively, by histopathological examination and amylase and lipase measurements.

It is known that increases in serum amylase and lipase activity is one of the characteristic features of AP. Amylase enzyme activity has been reported to start increasing by the 12th hour and peaks at the 24th hour [4]. In our study, the rats in the pancreatitis group had significantly higher amylase and lipase values compared to the control group (p < 0.001). Although the amylase and lipase levels in the treatment group were higher than the control group, the difference was not significant (p> 0.05).

Since it is now understood that ROS is one of the underlying pathophysiological factors in AP, many experimental studies have focused on using agents which enhance the effects of antioxidant enzymes such as SOD, GSH-Px, CAT with favorable results [4,15,16]. In this study, the oxidative stress markers (MDA) increased significantly in the cerulein-induced AP group (p<0.05), whereas there was no significant difference between the control group and the treatment group (p>0.05). Antioxidant enzyme GSH-Px was found to be significantly decreased in the pancreatitis group compared to the control group (p < 0.05). GSH-Px in the treatment group was found to be lower than in the control group and higher than in the pancreatitis group, but the difference was not statistically significant. There was no significant difference between the SOD and CAT values of the groups (p > 0.05). When oxidative stress markers and antioxidant enzyme activities were evaluated, there was significant difference between MDA and GSH-Px activities of the groups. However, this difference was not as apparent as that for the amylase and lipase enzyme levels and the histopathological evaluation. As stated by Szabolcs et al., this may be explained by the 10-fold lower basal scavenger enzyme levels in the pancreatic tissue compared to other tissues such as the liver. Low scavenger activity is shown as one of the causes of morbidity in patients with pancreatitis [4].

A recent study evaluated the effectiveness of SA in rats with AP and concluded that SA reduces tissue damage by increasing

antioxidant activity [2]. An important difference to note is that in our study rats received SA prior to the induction of AP, in order to establish the role of dietary phenolic acids in preventing AP or mitigating its severity. In this study, we observed that histopathological pancreatitis developed both in the pancreatitis group and the treatment group following cerulein administration. The apoptosis and the damage on the cellular level were significantly higher in the pancreatitis group (p < 0.001). Cellular damage in the treatment group was significantly lower than in the pancreatitis group (p < 0.05). When evaluated along with the results of previous studies, SA is thought to have both protective and preventive effects against AP.

Conclusions
Based on the results, this study shows that although dietary SA does not totally avert AP, it alleviates its severity both on enzymatic and histopathological levels. Dietary antioxidant agents may have protective effects against AP. This study could prompt the idea of dissemination of foods containing syringic acid, especially to the diets of patients who have frequent acute pancreatitis episodes. SA may be presented as tablets or capsules in the form of food fortification. We would like to note that for more precise results, studies involving intravenous administration of syringic acid at different doses are also needed. Also, who knows, it may be possible that the intravenous form could be used immediately at the beginning of AP.

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